Inhibition of Ribonucleotide Reductase by Gallium in Murine Leukemic L1210 Cells

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Abstract

Our previous studies of the mechanism of cell growth inhibition by gallium have suggested that the block in cellular iron uptake induced by transferrin-gallium results in an inhibition of the iron-dependent M2 subunit of ribonucleotide reductase. However, it is not known whether the inhibitory effect of gallium on ribonucleotide reductase is solely the result of limiting iron availability for enzyme activity or whether a direct effect of intracellular gallium on the enzyme is also involved. In the present study, utilizing a cell-free assay, we show that gallium nitrate directly inhibits CDP and ADP reductase activity. Inhibition of DNA synthesis by gallium nitrate thus appears to be due to a combination of a block in iron availability to ribonucleotide reductase and a direct inhibition of the enzyme by gallium.

Introduction

Gallium, a group IIIA metal resembles iron in that it binds to the iron transport protein transferrin (1), is taken up by cells through transferrin receptor-mediated endocytosis (2, 3) and is incorporated into the iron storage protein, ferritin (3, 4). In contrast to iron, however, gallium inhibits the growth of certain tumor cells in vitro and in vivo (5, 6). We have shown that transferrin-gallium blocks the incorporation of iron into human leukemic HL60 cells and that the growth-inhibitory effects of gallium can be reversed by transferrin-iron, iron salts, or hemin (7–9). Transferrin-gallium-treated cells display a decrease in intracellular deoxyribonucleotide pools and a diminution in the ESR spectroscopy signal of the iron-dependent M2 subunit of ribonucleotide reductase (9). While these studies suggest that gallium blocks DNA synthesis by inhibiting ribonucleotide reductase, it is unclear whether this effect results solely from a decrease in iron availability to the enzyme or whether a direct interaction of gallium with ribonucleotide reductase is also involved. To address this question, we have examined the effect of gallium on ribonucleotide reductase in a cell-free assay.

Materials and Methods

Reagents. Gallium nitrate was purchased from Alfa Products (Danvers, MA). [L-14C]CDP trisodium salt (532 mCi/mmol) and [2,8-3H]ADP (27.9 Ci/mmol) were purchased from DuPont (Wilmington, DE). Dowex 1-chloride, AMP-nitrophenyl phosphate, magnesium acetate, snake venom phosphodiesterase I (EC 3.1.4.1) from Croatalus adamanteus, and DTT were obtained from Sigma Chemical Company (St. Louis, MO).

Tissue Culture. Mouse leukemic L1210 cells were obtained from the American Type Culture Collection (Bethesda, MD) and were grown in suspension in RPMI 1640 supplemented with 10% horse serum. Cells were incubated at 37°C in an atmosphere of 5% CO2. For cell growth experiments, cells were plated at 5 x 104 cells/ml in the presence of increasing concentrations of gallium nitrate and cell growth was determined after 72 h of incubation.

Preparation of Cell-free Extracts. L1210 cells (106) in log growth phase (approximately 24 h after replating in fresh medium) were harvested and washed twice by centrifugation (1000 rpm for 10 min) with ice cold 150 mM NaCl-10 mM potassium phosphate buffer, pH 7.4. The final cell pellet was resuspended in 2 ml of Tris-DTT. Cells were disrupted using a Dounce homogenizer fitted with a motor-driven pestle (20 strokes) and the cell homogenate was centrifuged at 100,000 x g for 1 h. The supernatant was removed and passed through a 1-ml Dowex 1-acetate column to remove endogenous nucleotides. Proteins in the sample were then fractionated by the addition of solid ammonium sulfate (final concentration, 80%). Following centrifugation of the sample (40,000 x g for 30 min), the ammonium sulfate pellet was resuspended in Tris-DTT buffer and dialyzed against Tris-DTT buffer. The dialysate was concentrated to a volume of about 1.5 ml using a Centricon 30 concentrator (Amicon Corporation, Danvers, MA). An aliquot was removed for assay of protein content and the remainder of the sample was frozen in liquid nitrogen.

Protein Assay. Samples were assayed for total protein content using a BCA protein assay kit from Pierce (Rockford, IL).

CDP Reductase Assay. CDP reductase activity in the above cell-free preparation was assayed using a modification of a previously described method (10, 11). The assay mixture (total volume, 150 µl) contained 1.0 µmol sodium phosphate buffer (pH 7.0), 0.60 µmol magnesium acetate, 0.90 µmol DTT, 0.15 µmol AMP-nitrophenyl phosphate, 50–275 µg protein, 0–0.060 µmol gallium nitrate, 0.25–3.00 nmol [U-14C]CDP (specific activity, 20.4 µCi/µmol). After the mixture was incubated at 37°C for 30 min, the reaction was stopped by heating in a boiling water bath for 4 min. The samples were cooled and incubated for 2 h with snake venom phosphodiesterase mixture [1.2 µmol Tris (pH 8.7), 3.0 µmol MgCl2, 20 nmol dCMP, and 1 mg/ml snake venom phosphodiesterase]. Deoxycytidine was separated from cytidine using 1-ml Dowex 1-borate columns (10, 11).

ADP Reductase Assay. ADP reductase activity was assayed as described previously (12). The assay mixture (total volume, 150 µl) contained 0.15 µmol dGTP, 0.90 µmol DTT, 1 µmol sodium phosphate buffer (pH 7.0), 0.60 µmol magnesium acetate, 50–450 µg protein, 0–0.060 µmol gallium nitrate, 0.03–7.5 nmol [2,8-3H]ADP (specific activity 190 µCi/µmol). The reaction was allowed to proceed at 37°C for 30 min and was then stopped by placing the tubes in a boiling water bath for 4 min. After a 2-h incubation with phosphodiesterase mixture, the sample was applied to a Dowex 1-borate column to separate adenosine from deoxyadenosine (12).

Data Analysis. Enzyme kinetic data were fitted using the FORTRAN programs of Cleland (13). The log fit effectively assumes constant proportional error in velocities. Inhibition data fit best to Equation A for competitive inhibition, where v is the experimentally determined velocity, V is the maximum velocity, K is the Michaelis constant, A is the substrate concentration (ADP or CDP), I is the inhibitor concen-
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Trination (gallium nitrate) and $K_i$ is the slope inhibition constant.

$$\log v = \log \frac{VA}{[K(1 + I/K_i) + A]}$$ (A)

Results

Inhibition of Murine Leukemic L1210 Cell Growth by Gallium Nitrate. Fig. 1 shows the inhibition in growth of murine L1210 cells by gallium nitrate. A progressive decrease in cell growth was seen with increasing concentrations of gallium nitrate. The dose producing 50% inhibition of cell growth at 72 h was 21.1 µM. The effect of gallium nitrate during the 72-h incubation appeared to be cytostatic rather than cytocidal. Cells exposed to the highest concentration of gallium (100 µM) displayed a viability equivalent to that of control cells and a cell number close to initial plating density, thereby suggesting that cells had failed to progress through DNA synthesis and cell division following initial plating.

Inhibition of Ribonucleotide Reductase Activity by Gallium Nitrate. In five separate experiments we found that gallium nitrate, over a range of 5 µM to 1 mM, inhibited both CDP and ADP reductase activity. Table 1 exemplifies the result of this inhibition on CDP reductase activity. Lineweaver-Burk analysis of the inhibition of CDP reductase and ADP reductase activity by 100 µM gallium nitrate are shown in Figs. 2 and 3, respectively. The enzyme inhibition data fitted using the FORTRAN program of Cleland (13) fit best to a model of competitive inhibition. The fit to Equation A (see “Materials and Methods”) is an approximation, since the inhibition could also be due to a gallium-nucleotide complex itself (see “Discussion”). The $K_i$ for gallium nitrate versus ADP was 14.3 ± 3.0 (SE) µM for ADP reductase activity, while the $K_i$ for gallium nitrate versus CDP was 28.7 ± 3.4 µM for CDP reductase activity.

Discussion

The role of ribonucleotide reductase as a rate-limiting enzyme in DNA synthesis has made it an attractive target for antineoplastic chemotherapy (14). Mammalian ribonucleotide reductase, the enzyme responsible for the synthesis of deoxyribonucleotides, is comprised of two subunits termed M1 and M2 (15). The M1 subunit contains substrate and effector binding sites, while the M2 subunit contains non-heme iron and a tyrosyl free radical which produces a characteristic signal on ESR spectroscopy (16). The amplitude of this ESR signal in intact cells correlates with ribonucleotide reductase activity and increases as cells enter S phase (17, 18).

Our prior studies using HL60 cells suggested that transferrin-gallium inhibited DNA synthesis by inhibiting ribonucleotide reductase; however, these conclusions were based on measure-
mensions of intracellular deoxyribonucleotide pools and the tyrosyl radical ESR signal from the M2 subunit of ribonucleotide reductase (9). Since the activity of ribonucleotide reductase requires iron and since transferrin-gallium inhibits the cellular uptake of iron, it remained to be determined whether the inhibition of enzyme activity resulted solely from a decrease in iron availability to ribonucleotide reductase or whether it included a direct effect of gallium on the enzyme.

The present studies represent a continuation of our investigation into the mechanism of cell growth inhibition by gallium, and we now show that gallium is capable of directly blocking the activity of ribonucleotide reductase. Interestingly, the mechanism of action of gallium appears to involve competitive inhibition of substrate (CDP or ADP) interaction with the enzyme. It has been shown that gallium binds to ATP and ADP (19), and it appears reasonable to postulate that gallium may form gallium-CDP or gallium-ADP complexes which inhibit ribonucleotide reductase activity by blocking the binding of CDP or ADP to the enzyme.

These results, along with prior studies, suggest that gallium inhibits ribonucleotide reductase by at least two mechanisms. The first involves inhibition of cellular iron uptake at the level of the cell surface transferrin receptor with a subsequent decrease in the amount of iron available to the M2 subunit of the enzyme. Consistent with this mechanism is the finding that the M2 subunit ESR signal is markedly diminished following incubation of cells with gallium but can be fully restored within minutes by the addition of ferrous ammonium sulfate to cell lysates (8). The second mechanism of action of gallium, which is demonstrated in the current study, involves direct inhibition of ribonucleotide reductase activity by gallium.

Gallium nitrate is an effective agent in the treatment of hypercalcemia (20) and has antineoplastic activity against certain tumors in vivo (6). Studies of the intracellular targets of gallium are important since they provide a better understanding of the mechanism of action of this metal. Furthermore, because of certain similarities between gallium and iron, investigations into the interaction of gallium with cellular processes may also yield novel information about iron metabolism in neoplastic cells. Studies are in progress to further elucidate steps involved in the intracellular trafficking of gallium. Such investigations will undoubtedly provide a better understanding of the interaction of gallium with ribonucleotide reductase and other intracellular macromolecules.

References

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